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Comparison of Enhancement of GGTase-Positive Foci and Induction of Ornithine Decarboxylase in Rat Liver by Barbiturates

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16. ABSTRACT

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Comparison of enhancement of GGTase-positive foci and induction of ornithine decarboxylase in rat liver by barbiturates

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Abstract

The induction of ornithine decarboxylase (ODC) by barbiturates and the ability of barbiturates to enhance neoplastic progression of chemically initiated cancer was examined in rat liver. All seven barbiturates induced ODC with barbital (7.7 fold increase) and phenobarbital (5.7 fold increase) demonstrating the most potent activity. Maximum induction of ODC by phenobarbital was obtained in 18 h. Barbital (500–5000 p.p.m.) and phenobarbital (500 p.p.m.) administered in the drinking water enhanced the appearance of diethylnitrosamine (DENA)-initiated γ-glutamyltranspeptidase (GGTase)-positive foci. Amobarbital, hexabarbital and pentabarbital did not enhance the appearance of GGTase-positive foci. In the absence of previous initiation by DENA, the enhancing regimen of the barbiturates did not cause the appearance of GGTase-positive foci. Barbiturates induced ODC activity in rat liver and enhanced the incidence of DENA initiated GGTase-positive foci.

Introduction

Phenobarbital has been demonstrated to be a promoter of carcinogenesis in rat liver (1,2). Peraino *et al.*, (3,4) have demonstrated the enhancement by phenobarbital of the incidence of hepatomas in 2-acetylaminofluorene (AAF)*-initiated rats. Phenobarbital has also been shown to enhance the appearance of γ-glutamyltranspeptidase (GGTase)-positive foci in the liver of diethylnitrosamine (DENA) treated rats (1,2). These foci are putative preneoplastic lesions that are capable of progressing to hepatomas and hepatocellular carcinomas (2,5). The ability of other barbiturates to enhance the appearance of GGTase-positive foci in DENA-initiated rats is reported here.

The induction of ornithine decarboxylase (ODC, EC 4.1.1.7) by tumor promotoers has been demonstrated in mouse skin and has been proposed to be a molecular marker for tumor promoters (6). The cellular activity of ODC is a major site for the regulation of polyamine biosynthesis (7–9). The association of cellular ODC activity to cell proliferation has been reviewed by Russell and Durie (8) and Canellakis *et al.* (10). Elevated levels of ODC activity were found in rat liver undergoing rapid proliferation as the result of partial hepatectomy (11) or as the result of the administration of chemicals (11), drugs (11) or hormones (8). Induction of ODC would appear suitable for use as a molecular marker for the hyperplastic response of tumor promoters in the liver. In this communication, we report for various barbiturates the relationship between their enhancement of GGTase-positive foci and their ability to induce ODC.

*Abbreviations: AAF, 2-acetylaminofluorene; GGTase, γ-glutamyltranspeptidase; DENA, diethylnitrosamine; ODC, ornithine decarboxylase.

Materials and Methods

Animals and chemicals

Male Sprague-Dawley rats (Charles River Co., Portage, MI) weighing 175–225 g were used in these studies. They were fed Purina laboratory chow (Ralston Purina Co., St. Louis, MO) and given distilled water *ad libitum*.

γ-Glutamyl-4-methoxy-2-naphthylamide was purchased from Polysciences (Warrington, PA), DENA from Eastman Organic Chemical Co. (Rochester, NY), sodium phenobarbital from Mallinkrodt, Inc. (St. Louis, MO), the sodium salt of other barbiturates and NADP from Sigma Chemical Co. (St. Louis, MO), ACS from Amersham Corp. (Arlington Heights, IL), hyamine hydroxide from Packard Instrument Co. (Downers Grove, IL), O.C.T. compound and hematoxylin from Fisher Scientific, Co. (Pittsburgh, PA) and DL [1-^{14}C] ornithine (54.9 mCi/mmol) from New England Nuclear (Boston, MA).

GGTase-positive foci

Rats, in groups of 10, were initiated by intragastric administration of DENA (0.3 mmol/kg) in distilled water. One week later the rats started to receive the sodium salt of the barbiturates in their drinking water. Seven weeks after the start of the barbiturates, the rats were sacrificed, the liver excised and sections frozen in O.C.T. compound. Cyrostatic slices were obtained and stained for GGTase activity as previously described (1). Slices totaling at least 2 cm^2 from each animal were scanned for GGTase activity and foci containing at least nine nuclei scored as positive.

ODC assay

ODC activity was determined by the measurement of the amount of liberated $^{14}CO_2$ from DL-[1-^{14}C]ornithine essentially as described by Bethel and Pegg (12). Animals were always sacrificed between 9:00 and 10:00 a.m. by decapitation. All subsequent procedures were carried out at 0–4°C. Livers were homogenized in 1.0 volume of 0.1 M sodium phosphate containing 0.8 mM pyridoxal phosphate and 2.0 mM EDTA (pH 7.2). The postmicrosomal supernatant that was obtained by centrifugation at 144 000 x g for 60 min, served as the source of enzyme. The enzyme (100 μl; 0.4–0.6 mg protein) was added to Kontes incubation flasks containing 900 μl of the incubation medium consisting of 56 mM sodium phosphate (pH 7.5), 3.8 mM DTT, 2.3 mM EDTA, 8 mM DL-ornithine monohydrochloride, and 1.0 μCi of DL-[1-^{14}C]-ornithine (54.9 mCi/mmol). After incubation for 30 min at 37°C, the reaction was terminated by addition of 0.3 ml 5 M H_2SO_4 and allowed to stand for an additional 45 min while the liberated $^{14}CO_2$ was absorbed onto the hyamine hydroxide saturated filter paper. The filter paper with the $^{14}CO_2$ trapped onto it, was transferred to liquid scintillation counting vials containing 15.0 ml ACS and 0.41 ml of 0.5 N HCl. The radioactivity in the samples was measured in a Beckman Model 8100 liquid scintillation counter (Beckman Instrument Co., Fullerton, CA). ODC activity was expressed as pmol $^{14}CO_2$ liberated/30 min incubation/mg protein and exhibited a linear relationship of evoked CO_2 with time during the 30 min.

Results

Induction of ODC activity

The time course of phenobarbital induction of ODC activity in rat liver is depicted in Figure 1. Maximum enhancement of ODC activity occurred 18 h after a single dose of phenobarbital (80 mg/kg). The maximum level of activity was 9.68 ± 1.04 pmol $^{14}CO_2$ liberated/30 min/mg protein which represented a 5.7-fold enhancement of the control level. Therefore, 18 h was chosen for the length of treatment to be used in the comparison of the ability of other barbiturates to

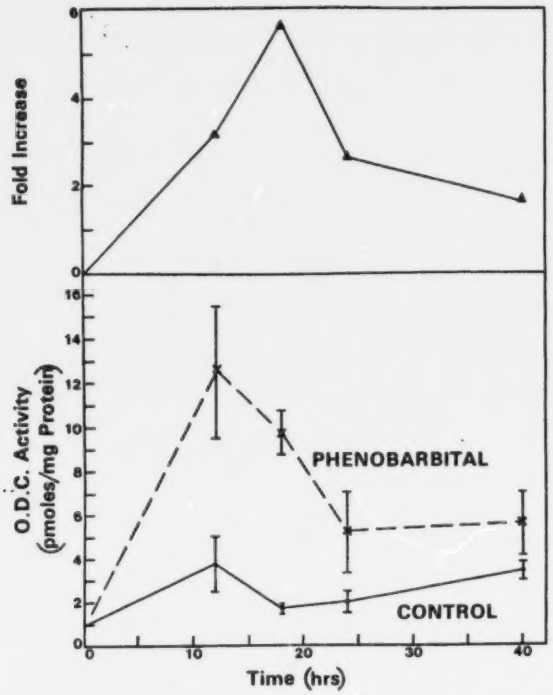

Fig. 1. Time course of phenobarbital induction of ODC. Groups of eight rats each were administered intragastrically with phenobarbital (80 mg/kg) and sacrificed at various times later. All animals were sacrificed between 9:00 and 10:00 a.m. ODC activity was measured in a cytosol fraction and expressed as pmol $^{14}CO_2$ liberated/30 min/mg protein. Results are means of eight rats each ± S.E.

enhance ODC activity.

Six other barbiturates were tested for their ability to induce ODC activity in rat liver. All six barbiturates produced an enhancement of ODC activity at 18 h (Table I). The doses of the barbiturates that were used represent 1/4 the LD_{50} and were chosen because they approximate the maximum dose suitable for tumor promotion studies. The potency of the individual barbiturates to enhance ODC activity varied. Barbital and phenobarbital at the doses tested were the most potent inducers of ODC activity; hexabarbital, pentabarbital and secobarbital possessed intermediate potency; and amobarbital and thiobarbital possessed weakest potency.

Enhancement of GGTase foci

The ability of barbital and phenobarbital to enhance the appearance of GGTase-positive foci was determined in experiment 1 (Table II). In rats initiated with DENA, both barbital and phenobarbital enhanced the appearance of GGTase-positive foci. The 500 p.p.m. dose of both barbiturates were of approximately equal potency resulting in 5.41 foci/cm² for barbital and 7.16 foci/cm² for phenobarbital. Doses of sodium barbital between 500 and 5000 p.p.m. were of equal potency in their ability to enhance the incidence of GGTase-positive foci. Nine of 20 rats that received 5000 p.p.m. barbital died before the end of the experiment and the rest appeared lethargic. This might explain in rats that received 5000 p.p.m. barbital the apparent, but statistically insignificant, decrease, compared to rats that received 500 or 2500 p.p.m., in the number of GGTase-positive foci. In animals, that were not previously initiated with DENA, barbital and phenobarbital did not result in an increased incidence of GGTase-positive foci. These results indicate that barbital and phenobarbital enhanced the appearance of previously initiated GGTase-positive foci but lacked the ability to initiate foci.

In experiment 2, the ability of amobarbital, barbital, hexobarbital and pentobarbital to enhance DENA initiated GGTase-positive foci was determined. Only barbital, which was the most potent inducer of ODC activity, produced a significant increase in the incidence of GGTase-positive foci. Though the incidence of GGTase-positive foci was increased after amobarbital, hexabarbital and pentabarbital, the increase was not statistically significant. Therefore, barbiturates that possessed weak to intermediate potency to induced ODC

Table I

Effect of barbiturates on rat liver ODC activity

Barbiturate	Dose mg/kg body wt.	Activity[a] pmol/30 min/ mg protein	Enhancement[b]
Amobarbital	75	4.19 ± 0.78[c]	2.5
Barbital	200	13.1 ± 3.0	7.7
Hexabarbital	200	5.53 ± 0.73	2.6
Pentabarbital	50	6.63 ± 1.33	3.9
Phenobarbital	100	9.68 ± 1.04	5.7
Secobarbital	50	5.14 ± 0.58	3.0
Thiobarbital	150	4.38 ± 0.73	2.6

[a]Activity is expressed as pmol $^{14}CO_2$ liberated/30 min/mg protein. [b]The saline vehicle control (1.0 ml/kg) was 1.70 ± 0.35. [c]Results are averages of 8 animals ± standard error.

Table II

Enhancement by barbiturates of rat liver GGTase-positive foci

Treatment		GGTase-positive foci
Initiation	Promotion	Number/cm²
Experiment 1		
DENA (0.3 mmol/kg)		
–	Control	0.98 ± 0.68[a]
+	Control	0.06 ± 0.06
	Barbital	
–	2500[b]	0.10 ± 0.10
–	5000	1.35 ± 0.94
+	500	5.41 ± 1.22[c]
+	2500	5.98 ± 1.18[c]
+	5000	3.98 ± 1.02
	Phenobarbital	
–	1000	0.33 ± 0.25
+	500	7.16 ± 0.94[c]
Experiment 2		
DENA (0.3 mmol/kg)		
+	Control	1.35 ± 0.31
+	Amobarbital (500)	2.32 ± 0.59
+	Barbital (500)	3.77 ± 1.06[d]
+	Hexobarbital (500)	2.06 ± 0.55
+	Phentobarbital (500)	2.03 ± 0.37

[a]Results are means ± S.E. of 10 rats each except 5000 p.p.m. barbital (–) DENA and (+) DENA which are results of the five and six surviving rats, respectively, of the original 10 rats/group. [b]Concentrations are p.p.m. in drinking (distilled) water. [c]Statistical evaluation was performed by a multiple comparison rank sums test of treatment vs. control (24). Results are different from both + and – DENA controls with $p < 0.01$. [d]Results are different from experiment 2 controls with $p < 0.05$.

activity, were at most weak enhancers of GGTase-positive foci.

Discussion

Induction of ODC has been proposed as a molecular marker for tumor promoters in mouse skin (6) and for hepatocarcinogenesis and the hyperplastic response of chemicals in rat liver (8,11,13,14). Phenobarbital is a tumor promoter in rat liver (1–3) that has been demonstrated upon acute administration to induce ODC (15). We were unable to demonstrate an increase in hepatic ODC activity for any of the groups in experiment 1 of the GGTase foci studies. It has been reported that the chronic administration of phenobarbital in the diet does not increase ODC activity (16). The asynchrony of the cell cycle coupled with the rapid turnover and large individual variation of ODC in the liver could have caused the chronic administration of phenobarbital in the drinking water and diet to result in a constant low level increase in enzyme activity that was below the sensitivity of detection. The demonstration of the induction of DNA replication in rat liver required the synchronization of cell proliferation by adopted feeding (17). Phenobarbital administered in the diet to DENA treated rats whose liver cell proliferation was synchronized by adopted feeding did slightly but significantly induce ODC activity (18). This increase was too small to be useful to rank barbiturates of possible lower inducing activity. Therefore, to rank substances according to their ability to induce ODC, it was necessary to acutely administer the barbiturates so as to synchronize the cells responding with an increase in ODC activity.

All seven barbiturates that were tested, possessed the ability to induce ODC activity. Barbital and phenobarbital possessed the greatest activity of 7.7 and 5.7-fold enhancement of control level, respectively. This difference in enhancement was not statistically significant. The five other barbiturates amobarbital, hexabarbital, pentabarbital, secobarbital, and thiobarbital produced a weak to intermediate response of 2.5–3.9-fold enhancement of ODC activity.

The use of foci containing hepatocytes of altered enzymatic activity, i.e., increased GGTase and decreased ATPase and glucose-6-phosphatase, to indicate the initiation of carcinogenesis has been reviewed (2,5,19,20). The foci appear to contain populations of preneoplastic cells that can progress to hepatomas and hepatocellular carcinomas (2,5,19,20). In animals previously initiated with a carcinogen, i.e., AAF or DENA, the ability of a second chemical administered at a later time, to enhance the appearance of GGTase-positive foci has been used as an indication that the second chemical possessed tumor promoting activity (2,20–23).

The ability of the barbiturates to enhance the incidence of DENA-initiated GGTase-positive foci was compared. Barbital and phenobarbital, the more potent inducers of ODC activity, were the only barbiturates that demonstrated the ability to enhance GGTase-positive foci. The ability of these two barbiturates to enhance the formation of GGTase-positive foci was not statistically different. The preneoplastic property and presumed precursor relationship to cancer of GGTase-positive foci (2,5,19,20) would indicate that barbital might also possess tumor promoting activity. The barbiturates that were less potent inducers of ODC activity, amobarbital, hexabarbital and pentabarbital, did not produce a statistically significant increase in the incidence of GGTase-positive foci. The inability of amobarbital to promote tumor formation was demonstrated by Peraino *et al.* (20). The inability to demonstrate an enhancement of GGTase-positive foci and tumor promotion while detecting a low level of induction of ODC activity, could be due to the greater sensitivity of the procedure employing acute administration of the barbiturate in the determination of the enhancement of ODC activity compared to the procedure used to determine enhancement of GGTase-positive foci. Therefore, weak inducers of ODC might possess enhancing activity below the sensitivity of the GGTase foci assay. In conclusion, the ability of a barbiturate to induce ODC would appear to be associated with its ability to enhance GGTase-positive foci. Thus, the ability to induce ODC in the liver appears to warrant further evaluation as a molecular marker for screening chemicals for the ability to enhance GGTase-positive foci and promote tumor formation.

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